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(54) Title: A METHOD OF SEQUENCING NUCLEIC ACIDS

(57) Abstract

A method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation, resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane. The filter membrane may retain nucleic acids by size exclusion and/or adsorb nucleic acids by surface binding.

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AMENDED CLAIMS

[received by the International Bureau on 21 December 1987 (21.12.87):
original claim 1 amended; remaining claims unchanged (1 page)]

1. A method for the sequencing of nucleic acid comprising recovering, from a host/vector system, vector particles containing a nucleic acid insert to be sequenced, treating the vector particles with a protease to digest proteinaceous contaminants, removing digestion products by ultrafiltration through a membrane which retains purified nucleic acid, and, thereafter processing the purified nucleic acid without removal from the membrane to produce a series of nucleic acid fragments for subsequent separation by electrophoresis.

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(54) Title: A METHOD OF SEQUENCING NUCLEIC ACIDS

(57) Abstract

A method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation, resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane. The filter membrane may retain nucleic acids by size exclusion and/or adsorb nucleic acids by surface binding.

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A method of sequencing nucleic acids.

The present invention relates to an improved method of sequencing nucleic acids, and particularly but not exclusively to an improved method of sequencing DNA.

The technique of nucleotide sequence analysis is of central importance in molecular biology and biotechnology. Contemporary methods of sequencing are mostly based on the high resolving power of polyacrylamide-gel electrophoresis. By this technique two oligonucleotides which differ in size by only a single nucleotide residue can be resolved by virtue of their differing relative interactions with the gel matrix. Alternative resolving methods are currently being investigated, for example column based systems such as high pressure liquid chromatography (HPLC).

In order to determine the sequence of nucleotides in a nucleic acid such as DNA a series of fragments are produced with one end in common and the other varying in position along the chain. A minimum of four types of series of fragments is produced, each group being terminated by or cleaved at one of the four possible bases by specific chemical or enzymic means.

There are two well known ways in which DNA fragments ending in or cleaved at each of the four bases are produced for sequence analysis. In the method described by Maxam and Gilbert (reference 1 and 2) single- or double-stranded DNA molecules are labelled with radioactive ^{32}P phosphate at a unique 5' or 3' terminus and the nucleotide chains are chemically modified at specific bases and then cleaved by alkaline or piperidine hydrolysis. The chain termination method of Sanger et al (reference 3 to 6) involves enzymic copying of single-stranded DNA fragments using a DNA polymerase to transcribe specific regions of the chain under controlled conditions.

In both the above described methods it is necessary to extract and purify the nucleic acids prior to initiating sequencing reactions. Conventionally RNA and DNA from tissues, cells, plasmids and viruses are extracted and purified by lysis or digestion of the protein coat (e.g. with a protease - such as proteinase K - which is able to digest the protein coat without degrading the nucleic acids) followed by extraction with solvent, such as phenol-chloroform, and precipitation with e.g. ethanol. Separation of nucleic acids from suspension in solvents is achieved by repeated centrifugation.

The phenol-chloroform reagent is a toxic corrosive liquid which is unpleasant to handle. After extraction with phenol-chloroform it is necessary to separate the aqueous from the organic phase. Centrifugation steps in the extraction/purification procedures are time consuming and tedious when carried out manually, and are difficult to automate satisfactorily.

The present invention provides an improved method of sequencing nucleic acids, such as DNA, which eliminates centrifugation steps during pre-sequencing purification, reduces manipulation of extracted products and is suitable for automation.

According to the invention, the improvement resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane.

The filter membrane may (a) retain nucleic acids by size exclusion and/or may (b) adsorb nucleic acids by surface binding. Examples of filter membranes of type (a) are anisotropic low adsorptive ultrafiltration membranes such as the type-YM membranes marketed by Amicon Corporation (US Patent No. 3488768). Examples of filter

membranes of type (b) are nitrocellulose membranes, NA45(TM), DE81(TM), Genescreen (TM), Hybond (TM) and the like; filter membranes having non-specific binding sites may be partially blocked with Bovine Serum Albumin (BSA) or other inert material.

An advantage of using ultrafiltration for separation and purification of nucleic acids is that the material collected on the membrane can be further treated in situ. For example, chemical or enzymic reagents can be applied to the membrane surface after filtration in order to prepare the trapped nucleic acids for sequencing.

The method of the invention can be used in an improved enzymic sequencing process including the following steps:-

1] A vector carrying a DNA insert to be sequenced is cultured in a host,

2] vector particles containing the DNA inserts are separated from the host by filtration,

3] a protease is applied which will digest the protein coat of the vector without degrading the DNA,

4] the DNA is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA,

5] primer is added and annealed to the extracted DNA on the membrane,

6] a polymerisation enzyme together with a suitable nucleotide mixture is applied to the DNA on the membrane, the enzyme being one which is capable of catalysing the faithful incorporation of nucleotides onto a primed template,

7] chase nucleotides are added if necessary in order to complete the polymerisation reaction,

8] the reaction is terminated and the newly synthesized DNA is disassociated from the template, and

9] a sample of DNA is loaded onto a polyacrylamide gel for electrophoresis, or onto an alternative separation system.

In the above described method any host/vector system can be used in which vector DNA is released from the host cell, generally in the form of a protein coated particle. The DNA could be single or double stranded; if the DNA is double stranded a denaturation step must be included before annealing in order to separate the strands. The chosen vector must have been manipulated so as to carry both the DNA fragment to be sequenced and the appropriate primer hybridisation site. A particularly suitable host is the bacterium E.Coli, and suitable vectors are bacteriophage M13, plasmid pEMBL and F1 derived vectors.

Examples of suitable protease are chymotrypsin, elastase, subtilisin and thermolysin. Residual protease activity can be terminated after the reaction has proceeded to completion by use of an ethanol wash or a protease inhibitor. Alternatively, the protease may have autolytic activity and hence be self-terminating. The catalysis enzyme is preferably a DNA polymerase such as a Klenow fragment of DNA polymerase I or reverse transcriptase. The enzyme must not have exonuclease activity which would digest the primer; if present, exonuclease activity can be blocked by the addition of a blocking group at the 5' end of the molecule.

If the above described method were adapted for sequencing with ribonucleotides, then ribonucleotides would be used instead of deoxyribonucleotides in the polymerisation step. In such a case an appropriate RNA polymerase would be used, possibly without the need

for a primer. Alternatively, if a suitable vector for RNA were developed, RNA could be used as the template.

One embodiment of the invention will now be described in detail, by way of example only. The Example illustrates an improved method of DNA sequencing using the chain termination method described by Sanger.

EXAMPLE

The method comprises the following steps :

1) Bacteriophage M13 with a DNA insert is cultured overnight in E.Coli in suitable culture medium.

2) The bacteria are separated from the phage by filtration with a 0.22 micron membrane, such as hydrophilic Durapore (Millipore). The bacteria remain on the filter and the DNA-carrying phage pass through the membrane into a collection vessel.

3) The phage are incubated with a protease, such as chymotrypsin, in order to digest the protein coat.

4) The DNA from the phage is separated from the digested protein by pressure activated ultrafiltration through a membrane which retains the DNA but not the digested protein. A preferred size-exclusion membrane is YM10 (Amicon) which retains globular molecules of size greater than 10 kDaltons. Alternatively, an adsorptive membrane such as nitrocellulose may be used. Non-specific binding of reagents to the filter can be prevented by the addition of 1 % bovine serum albumin.

5) The primer is added as a droplet or a spray to the DNA on the filter membrane and is annealed for 20 minutes at 55 to 65°C to allow specific hybridisation.

6) Premixed nucleotides and DNA polymerase I Klenow fragment are added as a droplet or a spray to the filter membrane and are incubated at 20°C for 15 minutes. The nucleotides are incorporated onto the primed template to synthesize DNA by chain extension. The nucleotides may include labelled nucleotides and chain terminating nucleotides.

7) Chase nucleotides are added if necessary.

8) A formamide droplet or spray is placed on the membrane and heated at 90°C for 5 minutes in order to terminate reaction and caused disassociation of newly synthesized DNA from the template.

9) A DNA sample is loaded onto a conventional polyacrylamide gel.

10) The DNA fragments are separated by electrophoresis.

REFERENCES

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2. Maxam, A.M. and Gilbert, W (1980), in Methods in Enzymology, (ed Wil, R), 68, p.499, Academic Press, London and New York.

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4. Sanger, F. and Coulson, A.R. (1975), J.Mol.Biol.,94, 441-448.

5. Sanger, F., Nicklen, S. and Coulson, A.R. (1977), Proc.Natl.Acad.Sci., USA,74, 5463-5467.

6. Air, G.M., Sanger, F. and Coulson, A.R. (1976), J.Mol.Biol.,108, 519-533.

C L A I M S :

1. An improved method of sequencing nucleic acids wherein the improvement resides in a purification step which comprises the pressure activated separation of DNA or RNA from a digest suspension by ultrafiltration using a filter membrane which retains the nucleic acids and presents them in a state ready for immediate commencement of sequence determination in situ on the membrane.

2. An improved enzymic sequencing process for DNA which includes the following steps :

i] a vector carrying a DNA insert to be sequenced is cultured in a host,

ii] vector particles containing the DNA inserts are separated from the host by filtration,

iii] a protease is applied which will digest the protein coat of the vector without degrading the DNA,

iv] the DNA is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA,

v] primer is added and annealed to the extracted DNA on the membrane,

vi] a polymerisation enzyme together with a suitable nucleotide mixture is applied to the DNA on the membrane, the enzyme being one which is capable of catalysing the faithful incorporation of nucleotides onto a primed template,

vii] chase nucleotides are added if necessary in order to complete the polymerisation reaction,

viii] the reaction is terminated and the newly synthesized DNA is disassociated from the template, and

ix] a sample of DNA is loaded onto a polyacrylamide gel for electrophoresis, or onto an alternative separation system.

3. An improved method of DNA sequencing using the chain termination method of Sanger, which includes the following steps :

a) Bacterophage M13 with a DNA insert is cultured in E.Coli in suitable culture medium,

b) the bacteria are separated from the phage by filtration with a hydrophilic membrane,

c) the phage are incubated with chymotrypsin protease,

d) the DNA from the phage is purified and concentrated by pressure activated ultrafiltration through a membrane which retains the DNA (e.g. a size-exclusion membrane which retains globular molecules of size greater than 10 kDaltons or an adsorptive membrane such as nitrocellulose),

e) primer is added as a droplet or a spray to the DNA on the filter membrane and is annealed for 20 minutes at 55 to 65°C,

f) premixed nucleotides and DNA polymerase I Klenow fragment are added as a droplet or a spray to the filter membrane and are incubated at 20°C for 15 minutes,

g) chase nucleotides are added if necessary,

h) a formamide droplet or spray is placed on the membrane and heated at 90°C for 5 minutes,

i) a DNA sample is loaded onto a polyacrylamide gel, and

j) DNA fragments are separated by electrophoresis.

4. An improved method of sequencing, substantially as hereinbefore described.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00384

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q 1/00 B 01 D 15/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0121778 (HOECHST) 17 October 1984 see page 3, line 8 - page 4, line 24; page 5, lines 1-6 --	1-4
X	EP, A, 0127737 (GAMBRO LUNDIA) 12 December 1984 see page 1, lines 17-26; page 2, lines 10-15; page 8, lines 26-31 --	1-4
X	Chemical Abstracts, volume 79, 15 October 1973, (Columbus, Ohio, US), C.W. Hancher et al.: "Evaluation of ultrafiltration membranes with biological macromolecules", see page 165, abstract 88983n, & Biotechnol. Bioeng. 1973, 15(4), 677-91 --------	1-4

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th September 1987	19 OCT 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00384

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/10/87

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0121778	17/10/84	DE-A-	3308932	13/09/84
		JP-A-	59173094	29/09/84
		US-A-	4623723	18/11/86
EP-A- 0127737	12/12/84	SE-A-	8302638	10/11/84
		JP-A-	59225197	18/12/84

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